DETECTION OF RED CELL ALLOANTIBODIES IN MULTIPLY TRANSFUSED THALASSEMIA MAJOR PATIENTS

Dissertation submitted to the

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1. INTRODUCTION

Thalassemia is a common condition, particularly in the meditermean region and southeast Asia. The term ‘thalassemia’ is used to describe a disorder with a siginificant decrease in the synthesis of one or more globin chains.[1]

Thalassemia is the most common single gene disorder in the world. Thalassemia has an autosomal recessive pattern of inheritance. It arises from mutation or deletion in one or more globin gene(s), leading to varying degrees of anemia that can range from insignificance to life threatening event.[2]

Life long red cell transfusion remains the main treatment for thalassemia. The recommended treatment for beta-thalassemia major is regular blood transfusion every three to four weeks, with a goal to correct anemia, to significantly suppress the hyperactive erythropoiesis and to inhibit gastrointestinal iron absorption[3]. The use of regular blood transfusion and iron chelation therapy has led thalassemia major from fatal disease in early childhood to a chronic illness with prolonged survival .[4]

Regular blood transfusion regimen is confronted with numerous complications. In almost every patient, the transfusion requirement slowly increases over years. Various factors contribute towards this

increased requirement include: development of hypersplenism, alloimmunisation against various blood group antigens, chronic infections, folate deficiency, aplastic crisis etc.[3]

Alloimmunisation to red cell antigens and transfusion transmissible infections are the major complications of transfusion particularly those who are chronically transfused. Some alloantibodies are hemolytic and may cause hemolytic transfusion reaction and may limit the possibility of further safe and effective transfusion, while others are clinically insignificant. Alloantibodies must be identified in the patient's serum before each transfusion so that compatible blood can be provided. The causes of alloimmunisation are not fully understood, however studies suggests that the recipients immune status, the difference in red cell phenotype between the donor and the recipient, the number of units the patients receive are the main contributing factors.[1]

It is a common scenario in India; thalassemia major patients receive suboptimal transfusion and chelation therapy due to number of reasons like, financial and social causes, lack of specialized centers, lack of safe blood transfusion services across the country. There are no adequate studies published on the adequacy of transfusion, morbidity and survival in thalassemia patients.

The center in which this study was conducted is one of the few centers in the country providing comprehensive management for

transfusion dependent thalassemia patients on a day care basis. This study is designed to determine the prevalence of alloimmunisation in multiply transfused thalassemia major patients and the factors that might contribute to their development.

2. AIMS AND OBJECTIVES

To study the prevalence of alloimmunisation to red cell antigens in multiply transfused thalassemia major patients.

3. REVIEW OF LITERATURE

Thomas Cooley, a Detroit pediatrician described a severe type of anemia in Italian children. He noted abundant nucleated red blood cells (RBCs) in peripheral blood about which he initially thought was erythroblastic anemia. Riette described Italian children with unexplained mild hypochromic microcytic anemia. Wintrobe and co-workers in United States reported a mild anemia in both parents of child with Cooley anemia. This anemia was similar to the one that Riette described in Italy. The severe form was labeled as thalassemia major and the mild form as thalassemia minor. The term Mediterranean anemia, which Whipple introduced, is misleading because this condition can be found in any part of the world.[5]

Thalassemias are inherited disorders of hemoglobin synthesis that result from an alteration in the globin chain production. A decrease in the production of certain globin chain(s) impedes hemoglobin synthesis and creates an imbalance with the other normally produced globin chains. This imbalance is the hallmark of all forms of thalassemia. For this reason, most thalassemias are not considered as hemoglobinopathies, because the globin chains are normal in structure and the defect is limited to a decreased production of these normal chains.[5]

Table 1 Human Hemoglobins[6]

Embryonic Hemoglobins	Fetal Hemoglobins	Adult Hemoglobins
Gower1	**HemoglobinF**	**HemoglobinA**
Zeta(2)	alpha(2)	alpha(2)
epsilon(2)	gamma(2)	beta(2)
Gower2		**HemoglobinA2**
alpha(2)		alpha(2)
epsilon(2)		delta(2)
Portland		
Zeta(2)		
gamma(2)		

The type of thalassemia usually carries the name of the under produced chain or chains. The reduction varies from slight decrease to a complete absence of production of these chains, Since β chain synthesis replaces γ chain synthesis during the first half of first year of life, severe forms of β thalassemia commonly present during the first year.

Table 2 Classification of β- Thalassemia[7]

Clinical Nomenclature	**Genotype**	**Disease**
Thalassemia major	Homozygous – β∫ Thalassemia(β∫/β∫) Homozygous – β+thalassemia(β+/β+)	Severe; requires blood transfusion
Thalassemia intermedia	β∫/β (β+/β+)	Severe, but does not require regular blood transfusions
Thalassemia minor	β∫/β β+/β	Asymptomatic with mild or absent anemia; red cell abnormalities seen

β – thalassemia is classified as in Table 3.2 above.

3.1 GEOGRAPHICAL DISTRIBUTION

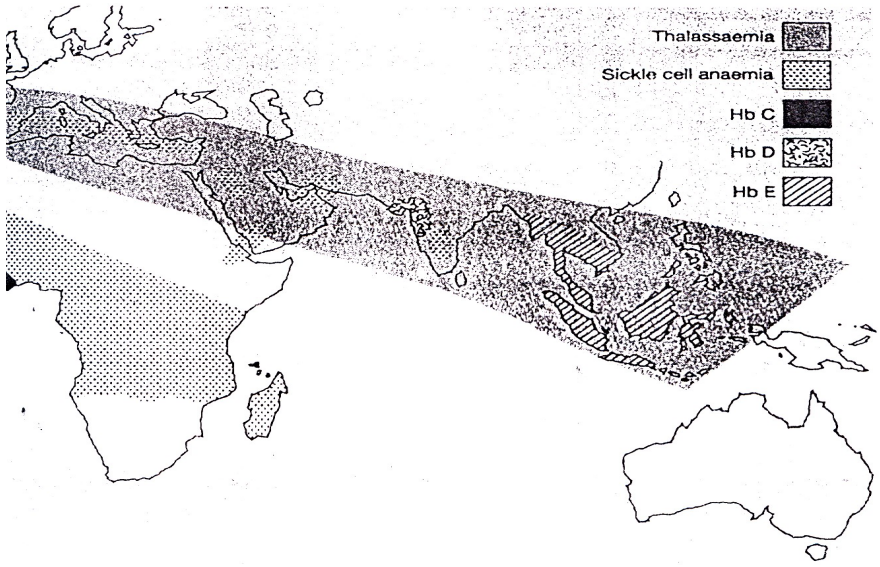


Figure 1 The geographic distribution of thalassemia and other hemoglobinopathies

Thalassemia was originally thought to be a disease limited to the Mediterranean region; however it is now known that it occurs widely throughout many parts of the world. Thalassemia has been identified across southern Europe from Portugal to Spain, Italy and Greece, as well as in a number of central European countries and parts of the former Soviet Union. Thalassemia also affects the Middle East through to Iran, Pakistan, India, Bangladesh, Thailand, Malaysia, Indonesia and southern China, as well as countries along the north coast of Africa and in South America.[8]

Population migration and intermarriage between different ethnic groups has introduced thalassemia in almost every country of the world, including northern Europe where thalassemia did not previously exist and where now it is becoming a major public health problem.[8]

3.2 THALASSEMIA IN INDIA

In India it is estimated that β thalassemia has a frequency at birth of 1: 2700, which means about 9000 cases of thalassemia major are born every year.[9]

The highest frequency of β thalassemia trait is reported in Assam, North eastern India, and Gujarat, followed by Sindh, Punjab, Tamil Nadu, and Maharashtra.[2]

3.3 GENETICS AND INHERITANCE

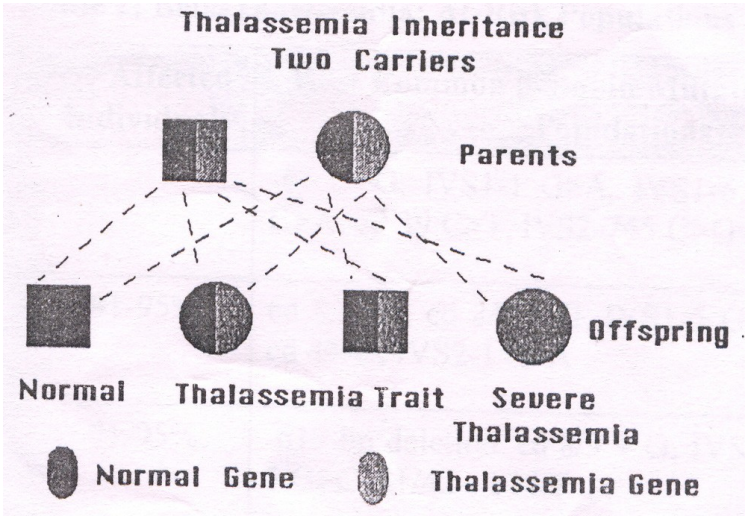


Figure 2 Inheritance of thalassemia genes

When both parents are carriers, then each child born to these parents has a one-in-four (25%) chance of being healthy (without the thalassemia gene), a one-in-two chance (50%) of being a carrier like its parents, and a one-in-four chance (25%) of having ß-thalassemia major.[10]

3.4 PATHOPHYSIOLOGY

The defect in □-thalassemia is in the switch from gamma ţo□ chain production with consequent precipitation of unpaired alpha chains in the developing red cells. This appears to be responsible for premature cell death in the bone marrow and hence for ineffective erythropoiesis and also for the abnormal red cells in the peripheral blood that are the cause of hypersplenism. In the bone marrow the myeloid : erythroid ratio is reversed and studies show an arrest of maturation in the early polychromatic erythroblasts.[11]

The effect of ineffective erythropoiesis on growth is generally seen in early childhood, and is quite different from the endocrine growth failure that occurs in older iron loaded patients. Early failure of growth occurs only in association with a grossly overactive marrow, when the transfusion scheme is too low, or when there is compensatory bone marrow expansion in the presence of hypersplenism.[11]

3.5 AGE AND SYMPTOMS OF PRESENTATION

Age at onset of symptoms varies significantly. In thalassemia severe cases are evident at birth, unexplained hypochromasia and microcytosis in a neonate are highly suggestive of the diagnosis. However in severe forms of β-thalassemia, symptoms may not be evident until the second half of the first year of life. Till that time the production of gamma globins and their incorporation into fetal hemoglobin can mask the condition.[5]

In reviewing 121 patients, Modell and Berdouckas, found that 60% presented within the first year: the mean age of presentation was 6 months.[12]

Table 3 Age at presentation of beta thalassemia

Age (years)	Thalassemia major
< 1yr	75 (62%)
1-2yrs	35 (29%)
>2yrs	11 (9%)
Total	121

Both sexes are equally affected in thalassemia. Thalassemia should be considered in any children with hypochromic microcytic anemia that does not respond to iron supplementation. The physician should always inquire about the patients ethnic back ground, family

history of hematological disorders, and dietary history.[5]

Occasionaly in children aged below 5 years, the condition may not be recognized because of the delay in cessation of HbF production. The affected children are usually pale, with feeding problems, irritability, recurrent infections, diarrhoea, and progressive enlargement of abdomen due to hepato splenomegaly.[5]

The bony changes may be severe resulting in a characteristic radiologic picture. The skull is large and deformed by frontal and posterior bossing. The zygomatic bones are prominent, the base of nose appears depressed and pneumatization of the sinuses are delayed. Over growth of the maxilla produces malocclusion. Metatarsal and metacarpal bones are the first to expand as a consequence of increased erythropoiesis. Poor growth is due to multiple factors, and it can be seen patients with well controlled disease and uncontrolled disease.[11]

The stigmata of severe untreated β-thalassemia major include.[10]

- severe anemia with hemoglobin level of 3-7g/dl
- Massive splenomegaly
- Severe growth retardation
- Bony abnormalities

3.6 LABORATORY DIAGNOSIS OF B-THALASSEMIA

MAJOR [13, 14]

- Anemia is severe with hemoglobin – 2-3g/dl
- Hematocrit and RBC counts are also decreased
- MCV,MCH,MCHC are all decreased
- Red cell Distribution width (RDW)- increased
- RBC histogram is shifted to the right and abnormal in shape

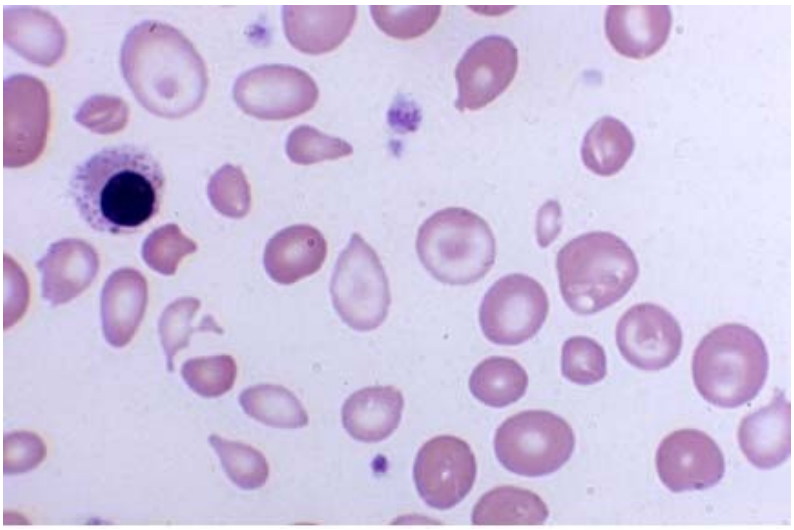


Figure 3 Peripheral blood smear in beta thalassemia major

- RBC's are markedly hypochromic, microcytic with aniso and poikilocytes.
- Target cells are many with schiztocytes leptocytes. (Large flat cells).
- Moderate basophilic stippling is seen.
- Platelet count is usually normal, unless the spleen is markedly

enlarged.

- Nucleated red blood cells are always seen.
- Reticulocyte count – relative increase 5-10%.

Bone marrow findings and iron status

- Bone marrow–erythroid, hyperplasia with dyserythropoiesis
- Stainable iron is increased and ring sideroblast may be seen.
- Serum iron- increased/ normal
- TIBC – decreased/normal
- Ferritin – increased/normal

Hemoglobin Electrophoresis

- HbA – decreased
- HbF – increased
- HbA2- variable.

3.7 BLOOD TRANSFUSION SUPPORT

Patients with thalassemia major require medical treatment and a regular blood transfusion. Children with hemoglobin values below 6–7 gm/dl should be observed very carefully at regular intervals with particular respect to their activity, growth and development, spleen size and early skeletal changes. Any infant showing deleterious effect of

anemia of this kind will require transfusion. Blood transfusion should be initiated at an early age when the child is symptomatic, after an initial period of observation to assess whether the child can maintain an acceptable level of hemoglobin without transfusion.[15]

Chronic regular blood transfusions provide patients with many benefits including revival of complication of anemia, elimination of ineffective erythropoiesis and its complications, allowance of normal or near normal growth and development and extension of patient's life span.[11]

Several blood transfusion regimens have been introduced. The regimen that attempts to maintain a pretransfusion hemoglobin level of 9-9.5g/dl seems more practical, less demanding and more cost effective than others .[15]

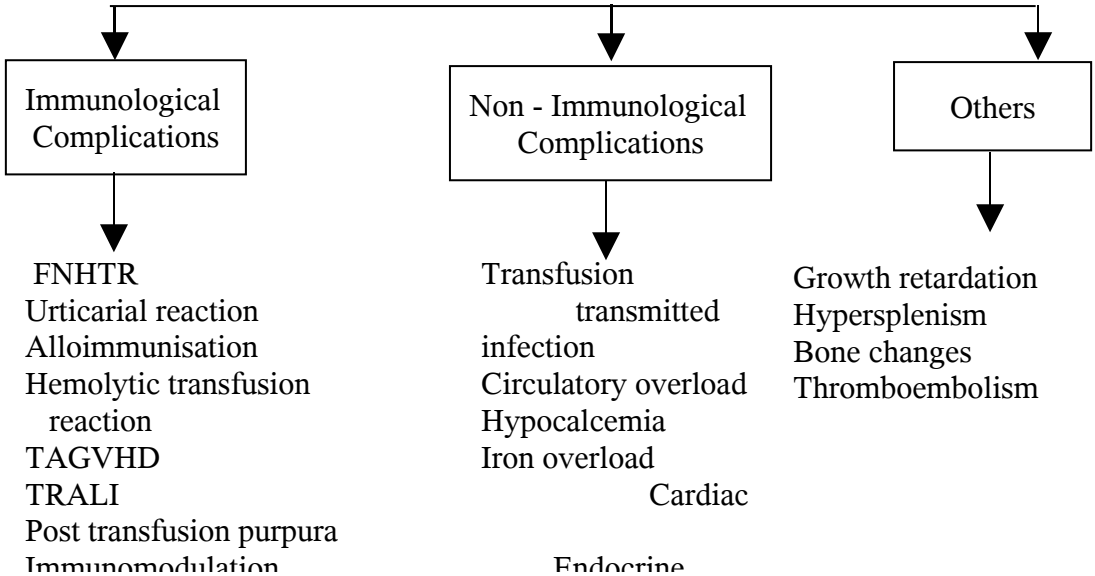
Thalassemia patients who are planned for chronic transfusions require a pretransfusion work up which should include red cell-phenotype, hepatitis B vaccination and hepatitis work up, iron and folate levels should also be measured.

Transfused blood should preferably be leukocyte poor, which are transfused at 10-15ml/kg at the rate of 5ml/kg/hr every 3-5 weeks. Some patients with very low pretransfusion hemoglobin may benefit from repeat transfusion on the following day. Patients with repeated Febrile

non-hemolytic transfusion reaction (FNHTRs) may benefit from premedication with acetaminophen and diphenhydramine hydrochloride before each transfusion. Some patients require saline washed or deglycerolized RBCs.[10] After many years of monitoring transfused patients, the inadequacy of transfusion alone as a therapy became clear, and the accumulation of iron from the transfused blood was slowly recognized. Today regular blood transfusion with well-monitored chelation therapy has become the standard therapy and has drastically changed the outlook of these populations of patients.

3.8 COMPLICATIONS OF BLOOD TRANSFUSION

Thalassemia patients are prone to develop complications due to chronic blood transfusion. The major complications of blood transfusion mainly divided into immunological and non-immunological.



3.8 RED CELL ALLOIMMUNIZATION

Red cell antibodies are detectable in up to 2.6% of the general population, at higher rates among individuals who are chronically transfused, and rarely among infants younger than 4 months. The prevalence among different patient groups varies from 18% to 47% in patients with sickle cell anemia, 5% to 11% in patients with thalassemia.[16]

The risk of red cell antibody formation depends on a myriad of factors including the patient's underlying illness, genetic predisposition, immune status, degree and duration of antigen exposure (e.g., total number of transfusions), and degree of antigen disparity with the blood donor.[17]

Delayed hemolytic transfusion reactions due to non-ABO incompatibility are largely prevented with accurate antibody identification and component phenotyping during compatibility testing. However, recently transfused red cells may become incompatible if red cell antibodies quickly develop because of transfusion in a previously sensitized patient (anamnestic response). Despite the coincidence of antigen positive transfused red cells and incompatible antibody, investigation often reveals no evidence of accelerated red cell destruction; in these cases, the findings have been termed a delayed serologic transfusion reaction (DSTR).[18]

The diagnosis of a DHTR requires clinical or laboratory evidence that the newly discovered red cell alloantibody is actually causing hemolysis (e.g., fever, falling hematocrit, and jaundice).

Implicated antibodies in DHTRs are typically IgG, reactive at 37^0C that fix complement (C3d) on red cells, antibodies to various antigens of the Rh, kell, Kidd and Duffy (Fy^a) system are more likely to cause delayed hemolytic reactions than other antibody specificities. The progressive removal of portions of the red cell membrane by phagocytic cells in the spleen results in the appearance of spherocytes in the peripheral circulation. Accelerated destruction of transfused red cells is also evident by reticulocytosis, unconjugated hyperbilirubinemia, and increased serum lactate dehydrogenase. Prolonged intervals between the initial and subsequent red cell transfusion predispose to DHTRs because the antibody titer may decrease so that it is no longer detectable in routine pretransfusion screening tests. Delayed hemolytic transfusion reactions occur 2 days to 2 weeks after re-exposure to the implicated antigen and are associated with mild hemolysis of transfused red cells in most clinical settings.[17]

Bystander hemolysis of the patient's own red cells as well as transfused red cells may result from complement activation and deposition of C3d on autologous red cells.

Standard blood bank practices are designed to prevent DHTRs by

accurate record keeping of prior red cell alloantibodies and avoidance of re-exposure to implicated red cell antigens for all future transfusions. As a further precaution in repeatedly and chronically transfused patients, investigation should include obtaining an accurate patient history regarding previous transfusions at other institutions and any clinically significant serologic findings. Primary red cell alloimmunisation may be prevented by avoiding unnecessary transfusion and by minimizing the potential for blood group antigen incompatibility between the blood donor and transfusion recipient.[17] Because patients with thalassemia are at the greatest risk of serious transfusion complications and are often dependent on long-term red cell transfusion, additional measures are warranted to prevent red cell alloimmunisation and prevent DHTRs. Recent clinical practice guidelines advocate that all patients with thalassemia major and sickle cell disease, should have their extended red cell antigen phenotype (ABO, Rh, Kell, Kidd, Duffy, Lewis, and MNSs blood group systems) determined before they start transfusion therapy, and they should receive ABO / Rh type-specific units that are phenotypically matched for C, E, and K1.[19] More extensive antigen matching is recommended for those patients who develop red cell alloantibodies.

3.9 TRANSFUSION TRANSMISSIBLE INFECTIONS

Transfusion Transmissible Infections (TTI) like hepatitis B virus

(HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV) etc, are dreaded complications of transfusion.[20]

The risks of infectious disease transmission by transfusion were reduced through introduction and progressive improvements in donor screening and infectious disease testing.[16] The discovery in the mid 1980's that acquired immunodeficiency syndrome (AIDS) could be transmitted by transfusion, heightened public concern about blood safety. Over the past decade efforts have been made to quantify the risk of transfusion transmitted infectious disease.[20]

In India it is mandatory to screen donated blood for, HBsAg, anti HIV 1&2 (since 1991), anti HCV (since 2000), syphilis and malaria

The greatest threat to the safety of the blood supply is the donation of blood by seronegative donors during the infectious window period when the donors are undergoing seroconversion. [20]

In addition to the above, other viruses like Cytomegalovirus (CMV), Epstein-Barr virus, Human Herpes-6, Human Herpes-7, Human Herpes-8, are associated with leukocyte contamination during transfusion. The human T-cell leukemia/lymphoma virus (types 1&2) targets.

T-lymphocytes and are solely transmitted through cellular

products. Primary toxoplasmosis has been reported to be transmitted through whole blood. Variant Creutzfeldt- Jacob disease (vCJD), SEN virus, west Nile virus, TT virus are recently feared to be transmitted by transfusions.[21] Yersinia enterocolitica is increasingly recognized as a cause of morbidity and mortality in patients with iron overload especially those with homozygous beta-thalassemia. [22]

The seroconversion rates among these donors were highest for HBV (9.80) 100, 0000 person years, followed by HCV (4.32 per 100, 0000), HIV (3.37 per 100,000), and HTLV (1.12 per 100,000). These rates are lower than those in the general population, a finding that confirm the effectiveness of donor education and history taking procedures.[20]

Implementation of nucleic acid testing (NAT) will reduce the residual risk of HIV by 50% and the risks HCV and HBV infection can be expected to be reduced by 72 and 42 percent respectively .Although new techniques of testing will bring the goal close to zero risk, it is unlikely that any test or combination of tests will be 100 percent effective in detecting window period infections. It is also important to recognize that new, direct viral detection tests will supplement existing screening assays rather than to replace them.[20] The levels of virus decline after seroconversion, a small percentage of antibody positive donors will test negative for viral antigens and nucleic acids yet still be

infectious. Therefore the yield and cost effectiveness of new, direct assays for viral detection is low and decisions about their implementation will be difficult.[20]

3.10 OTHER COMPLICATION

As the risks of infectious disease transmission by transfusion were reduced through the introduction of progressive improvement in donor screening, non infectious complications although uncommon, now account for most of the significant morbidity and mortality from blood transfusion in developed countries.[16] Non infectious complications now far exceed the risk of human immunodeficiency virus or other infectious diseases by order of magnitude. Iron induced heart failure and arrhythmias are the most common causes of death in patients with thalassemia major accounting for 70% of death.[23]

Iron chelating agents like desferrioxamine and deferiprone were used for two decades. The newly synthetic iron chelator deferasirox (ICL-670, Exjade) is currently used for iron chelation in thalassemic patients. Deferasirox is given in once daily dose of 20 mg/kg body weight. The drug is well tolerated and is highly selective for iron and does not induce the excretion of zinc or copper. Iron excretion is predominantly fecal.[23]

Endocrine complications are mostly due to iron overload and

deposition of iron in organs and tissues. Patients with thalassemia major frequently inhabit features of diabetes mellitus due to iron deposition in the pancreas.[21] Growth retardation is caused in part by the diversion of caloric resources for erythropoiesis as well as by chronic anemia. Hyper transfusion usually restores normal growth, but unless chelation therapy is initiated in early life, these patients rarely grow normally. Excessive chelation with DFO may also cause growth retardation. Impaired Growth hormone production or deficiency of somatomedin by the hemosiderotic liver is the direct cause of growth retardation. Involvement of the adrenal and thyroid glands also contribute to growth failure.[24]

Adult patients with thalassemia major are known have low fertility due to hypogonodotrophic hypogonadism. Both primary and secondary sexual characteristics are usually delayed in both males and females.

Females are frequently oligomenorrhic and amenorrhoeic Pregnancy complications seen are frequently due to endocrine and cardiac complications. However case reports have demonstrated successful pregnancy and delivery of healthy babies.[5] Early intervention with adequate transfusion support along with iron chelation and hormonal therapy prevents permanent damage and preserve fertility.[24,21]

3.11 ROLE OF SPLENECTOMY

The constant bombardment of the reticuloendothelial system with abnormal red cells and development of the extramedullary erythropoiesis are the important causes of Splenomegaly. Alloimmunisation and autoimmunisation to red cell antigens also causes splenomegaly. Patients present with physical discomfort because of the enlarged spleen. Several studies have reported a decreased red cell survival in thalassemic children with enlarged spleen.[25]

Large spleen is associated with inadequate rise in post transfusion hemoglobin and increased blood consumption there by to iron over load. Splenectomy is considered when

1) Transfusion requirement exceeds 200-220 ml/kg/year, to maintain a pretransfusion hemoglobin of about 10 gm/dl
2) Leucopenia
3) Thrombocytopenia
4) A very large spleen with a possibility of splenic rupture.[5,26]

3.12 DIET

A normal diet is recommended with small doses folic acid, ascorbic acid, and alphatocopherol (vitE) is sufficient. Iron supplements should not be given, and foods rich in iron should be avoided. Drinking coffee or tea with food has been shown to decrease iron absorption from gut.[5]

3.13 HEMATOPOIETIC STEM CELL TRANSPLANTATION

Hematopoietic stem cell transplantation (HSCT) is the only recommended curative treatment for thalassemia major. Care givers of patients with thalassemia are frequently confronted with a choice between standard therapy and HSCT. The 15yr cardiac disease free survival rate for patients receiving standard therapy exceeds 90% and is similar for those without risk factors who have under gone HSCT.[5]

Even though blood transfusion is not required after a successful transplant, certain individuals may need continued chelation therapy to remove excessive iron. The optimal time to start such treatment, is a year after the successful HSCT. Long term outcome for transplant, including fertility is not known. The cost of long term standard therapy is known to be higher than the cost of transplant. The possibility of developing cancer is not known.[5]

3.14 NEWER THERAPIES

Induction of fetal hemoglobin synthesis by drugs like, 5-azacytidine which can reduce the severity of beta-thalassemia by improving the imbalance between alpha and non alpha globins are under clinical trials.[27] The possibility of correction of the molecular defect in hematopoietic stem cell by transfer of normal gene via a suitable vector or by homologous recombination is being actively investigated. The most promising results in mouse model have been obtained with lentiviral vectors.[28]

4. PATIENTS AND METHODS

This prospective study was conducted over one year period from 2007-2008 at Voluntary Health Services Hospital, Tharamani, Chennai on 51 registered Thalassemia Major patients. The study was approved by the Ethical committee, The Tamil Nadu Dr.M.G.R. Medical University. Written consent from patients was also obtained. Thalassemia major patients treated with ten or more transfusions were included in the study. Diagnosis of thalassemia was confirmed by standard Hb electrophoresis.

Clinical transfusion record of patients who fulfilled the criteria were collected and entered in the proforma, with special reference to age ,age at the time of diagnosis, frequency of transfusion ,present clinical status, any increase in transfusion requirements, ethnicity, status of spleen, TTI screening ,iron chelation allo antibody screen etc.

Blood samples were obtained for the detection of anti red cell alloantibodies. Serum was separated using was standard blood bank method and stored in labeled tubes at -20° C, till the tests were performed in batches.

4.1 INCLUSION CRITERIA

1. Registered patients of thalassemia major confirmed by the Hemoglobin electrophoresis.
2. Patients who had at least ten transfusions before the commencement of study.
3. Patients who were on regular transfusion regimen.
4. Patients who were willing to participate in the study by giving written consent.

4.2 EXCLUSION CRITERIA

1. Patients other than thalassemia major like thalassemia minor and intermedia and all other variants of thalassemia.
2. Patients who were not under regular transfusion regimen.

4.3 MATERIALS AND METHODS

- Screening cells
- Test Tubes
- Anti Human Globulin (AHG)
- LISS
- 22% BSA
- Dry air incubator

- Micropipette
- Disposable tips

Antibody screening is done by conventional tube technique using 22% bovine serum albumin, and low ionic strength saline (LISS) as enhancement reagents. A three cell panel from Dia-Med was used for antibody screening.

4.4 SALINE INDIRECT ANTI GLOBULIN TEST

1. Add 2drops of serum to properly labeled tubes.
2. Add 1 drop of 2-5% saline suspended reagent cells to each tube and mix.
3. Centrifuge and observe for hemolysis and agglutination. Grade and read the results.
4. Incubate at 37° C for 30 min, centrifuge and observe for hemolysis and agglutination. Grade and record the results.
5. Wash the cells three times with saline and completely decant the final wash
6. Add a drop of AHG (Anti Human Globulin) to the dry cell button and mix well centrifuge and observe for agglutination. Grade and record the results
7. Confirm the validity of the negative tests by adding IgG coated red cells

4.5 ALBUMIN OR LISS-ADDITIVE INDIRECT ANTIGLOBIN TEST

1. Add 2 drops of serum to properly labeled tubes.
2. Add equal volume of 22% bovine albumin or LISS additive.
3. Add 1drop of 2-5 % saline suspended reagent cells to each tube and mix
4. For albumin incubate at 37° C for 30minutes, for LISS incubate for 15 minutes
5. Centrifuge and observe for hemolysis and agglutination. Grade and record the results. Follow steps 5,6,and 7 as in the earlier procedure

4.6 INTERPRETATION OF RESULTS

The results were interpreted as positive or negative based on the presence or absence of agglutination. Both positive and negative reactions were important in antibody identification. Positive reaction was compared to the antigen pattern expressed by the panel cells .Negative reactions are also important as they allowed tentative exclusions on non-reactive cells.

4.7 EXCLUSION OR CROSSING OUT

The first approach to the interpretation of panel results was to exclude specificities based on non-reactivity with the serum tested. Such a system is sometimes referred to as a “cross-out” or “rule-out” method. Once results had been recorded on the worksheet, the antigen profile of the first non-reactive cell was examined. If an antigen was present on the cell and the serum did not react, the presence of the corresponding antibody could be at least tentatively ruled out. After all antigens present on that cell had been crossed off, interpretation proceeded with the other non-reactive cells and additional specificities were excluded.

Next, the cells reactive with the serum were evaluated. The pattern of reactivity of the non-excluded specificity was compared to the pattern of reactivity obtained with the test serum. If there was a pattern that matched exactly, that was most likely the specificity of the antibody in the serum however, if there were remaining specificities that had not been excluded, additional testing was needed to eliminate remaining possibilities and to confirm the specificity identified. This requires testing the serum against additional cells.

Once tentative identification had been established, testing the serum against cells selected for specific antigenic characteristic gave more information than using an additional, unmodified panel. When the exclusion approach identified simple antibody specificities, it was

considered only a provisional step, particularly if the cross-out completed was based on non-reactivity of cells with heterozygous expression of an antigen.

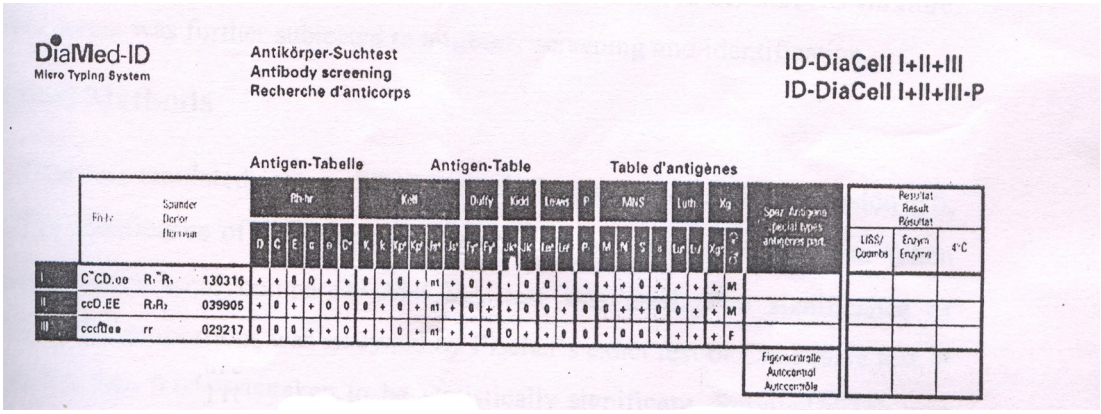


Figure 4 Antibody screening three –cell panel

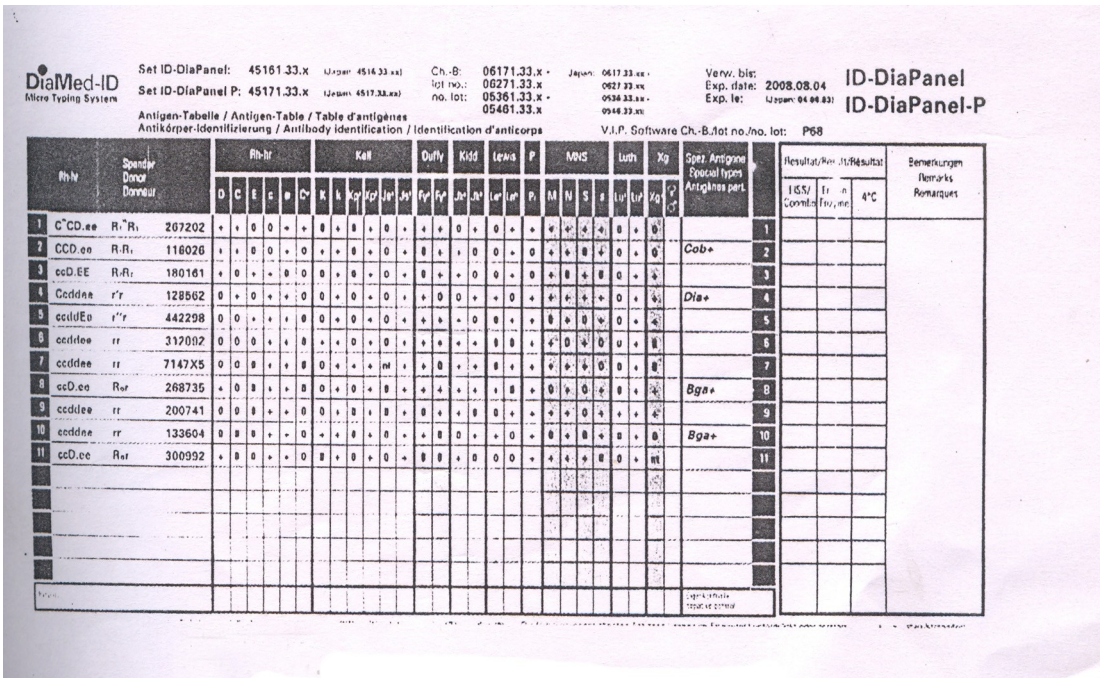


Figure 5 Antibody Identification 11 –cell panel

5. RESULTS

A total of 51 thalassemia major patients undergoing regular transfusion therapy were analysed as follows :

5.1 DEMOGRAPHIC DETAILS

- Age distribution
- Gender distribution
- Ethnicity
- Distribution of blood group.

5.2 CLINICAL FEATURES

5.3 ANALYSIS OF TRANSFUSION

- Age at diagnosis and start of first blood transfusion
- Transfusion interval.
- Transfusion reactions
- Transfusion transmissible infections
- Status of spleen.

5.1 DEMOGRAPHIC DETAILS

Table 4 Age Distribution of the Study Group

Age group in years	Number of patients	Percent %
< 5	11	21.6
6 – 10	22	43.1
11 – 15	8	15.7
16 – 20	7	13.7
21 – 25	1	2.0
25 – 30	2	3.9
TOTAL	51	100.0

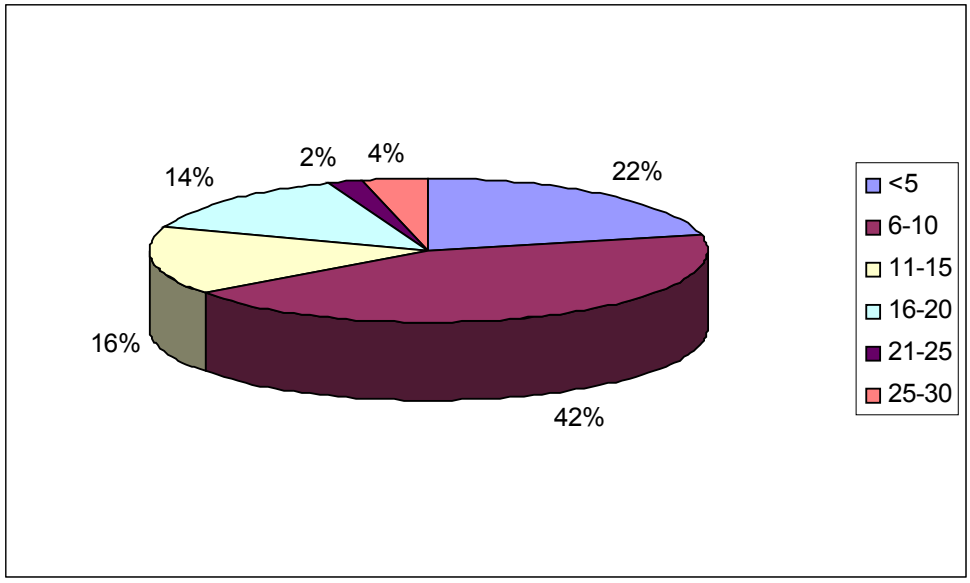
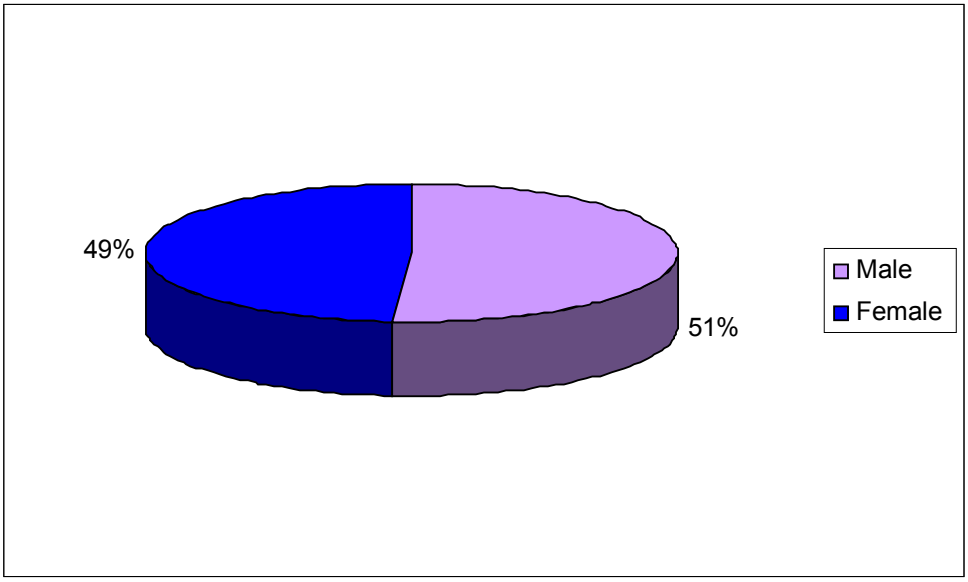

22%
42%
16%
14%
2%
4%
<5
6-10
11-15
16-20
21-25
25-30

Table 5 Gender Distribution of the Study Group

Sex	Number of Patients	Percent %
Male	26.0	51.0
Female	25.0	49.0
Total	51.0	100.0

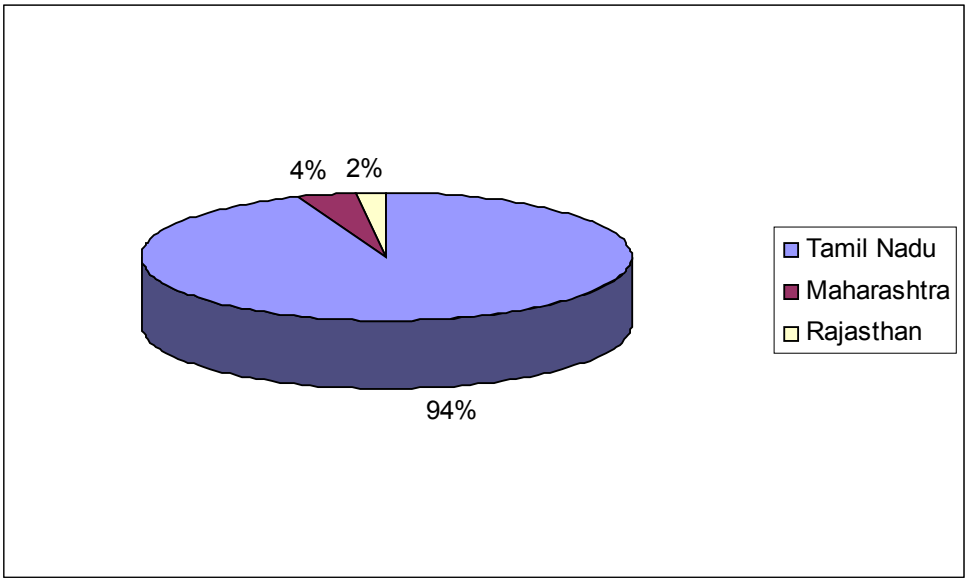


From the above table 51% of the patients were male and 49% of the study patients were female. There was no gender difference in the distribution of the disease.

The majority of the patients fall between the age group 6 – 10 years. The mean age of the study group is 9.84 years.

Table 6 Ethnicity of the Study Group

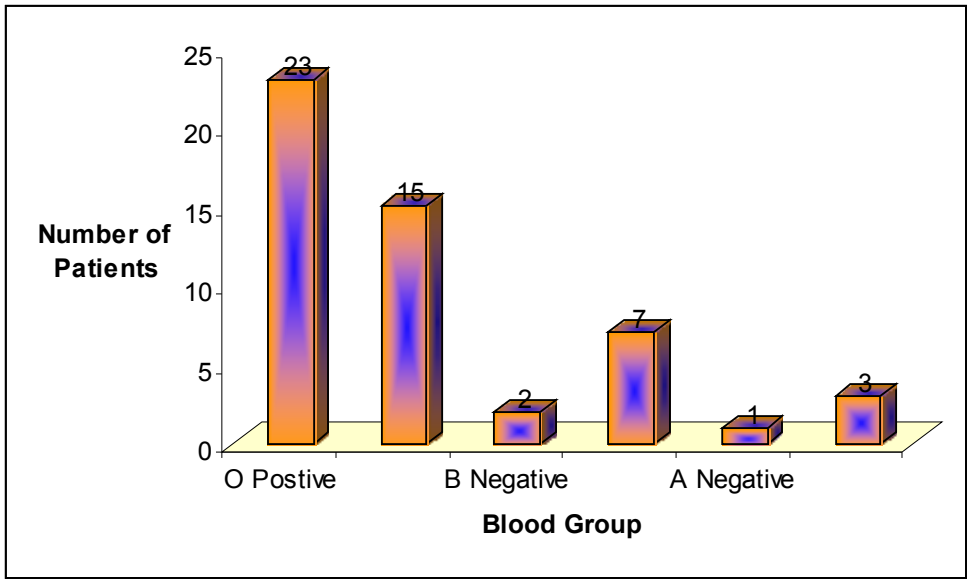
Ethnicity	Frequency	Percent %
Tamil Nadu	48	94.1
Maharashtra	2	3.9
Rajasthan	1	2.0
Total	51	100.0



94% of patients in the study group were from Tamil Nadu, two patients (3.9%) were from Maharashtra and 1 (2%) patient from Rajasthan.

Table 7 Distribution Of Blood Group

Blood Group	Number of Patients	Percent %
O Positive	23	45.1
O Negative	0	0
B Positive	15	29.4
B Negative	02	03.9
A Positive	07	13.7
A Negative	01	02.0
AB Positive	03	05.9
Total	51	100.0



Most of the study population belongs to O Positive blood group (45%)

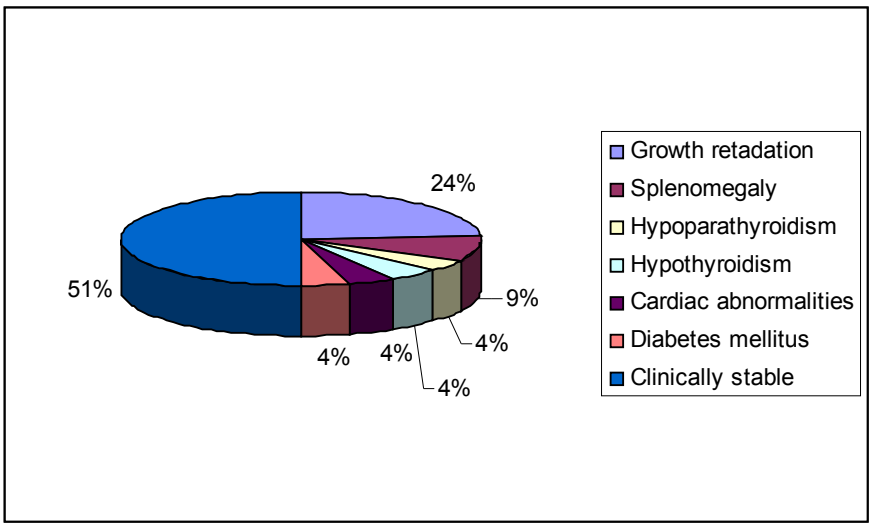
5.2 CLINICAL FEATURES

The Thalassemic children presented with the following clinical features

- Growth retardation
- Splenomegaly
- Hypoparathyroidism
- Hypothyroidism
- Cardiac abnormalities
- Diabetes mellitus
- Clinically stable.

Table 8 Present Clinical Status

Clinical Features	Number of patients	Percent %
Growth retardation	11	21.5
Splenomegaly	4	7.8
Hypoparathyroidism	2	3.9
Hypothyroidism	2	3.9
Cardiac abnormalities	2	3.9
Diabetes mellitus	2	3.9
Clinically stable	23	45



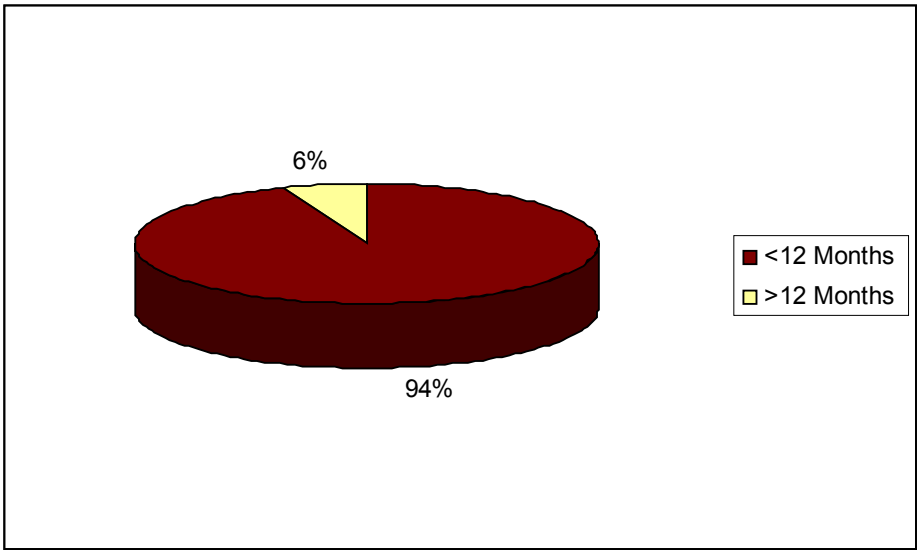
Analysis of Transfusion

Thalassemic children who received regular transfusion were analysed as follows.

- Age at Diagnosis and start of first blood transfusion
- Transfusion Interval
- Transfusion Reaction
- Transfusion Transmitted infection
- Splenectomy

Table 9 Age at Diagnosis and Start of First Blood Transfusion

Age in months	Number of patients	Percent %
< 12 MONTHS	48	94.1
> 12 MONTHS	03	05.9
Total	**51**	**100**

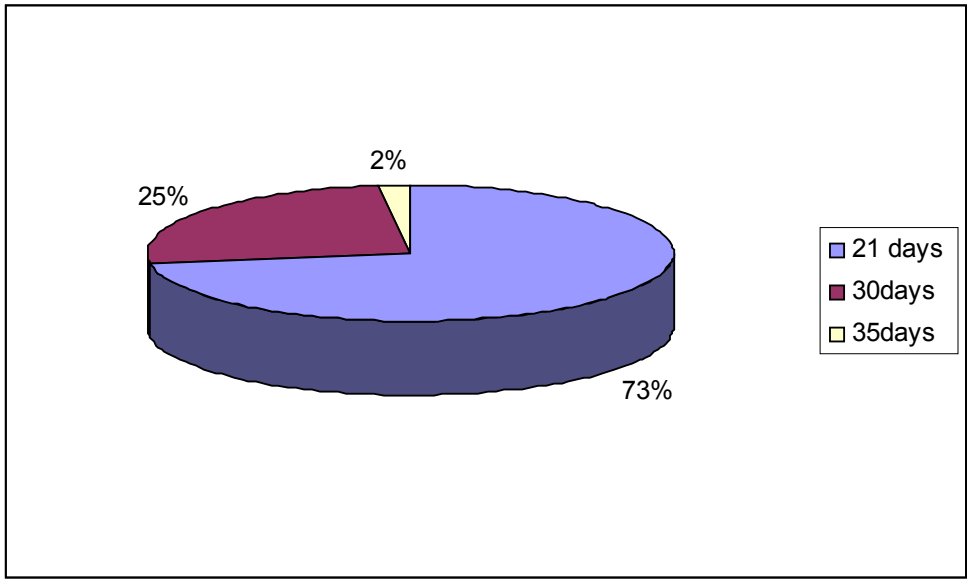


Majority (94%) presented with clinical symptoms and had their first transfusion before one year of age.

Table 10 Transfusion Interval

Tranfsusion frequency in days	Number of patients	Percent %

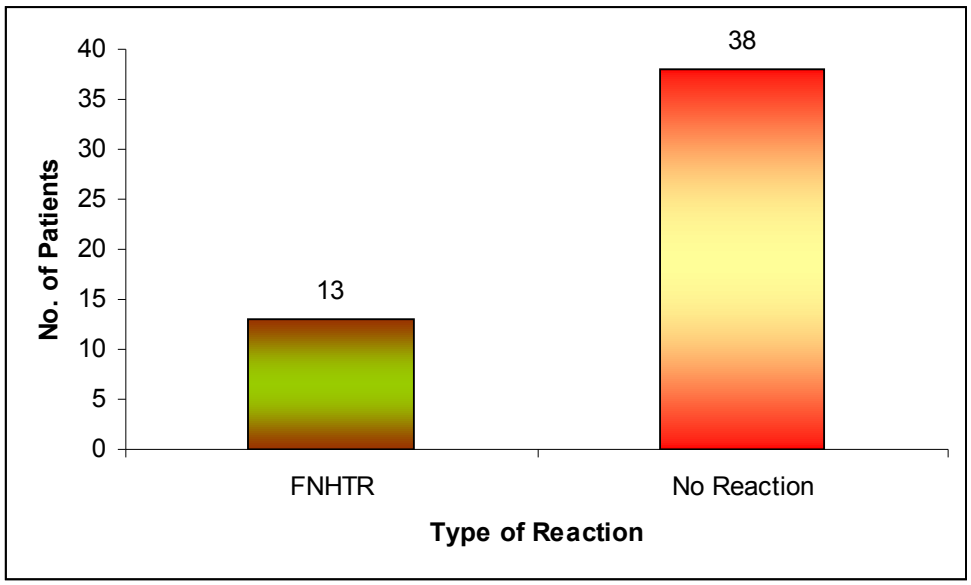
21	37	72.6
30	13	25.5
35	1	2.0
TOTAL	**51**	**100.0**



The transfusion interval varied widely in the study group ranging from 15 days to 35 days. The mean transfusion interval was 28.6 days. Most of the patients (70.6%) had their next transfusion every 21 days.

Table 11 Transfusion Reactions

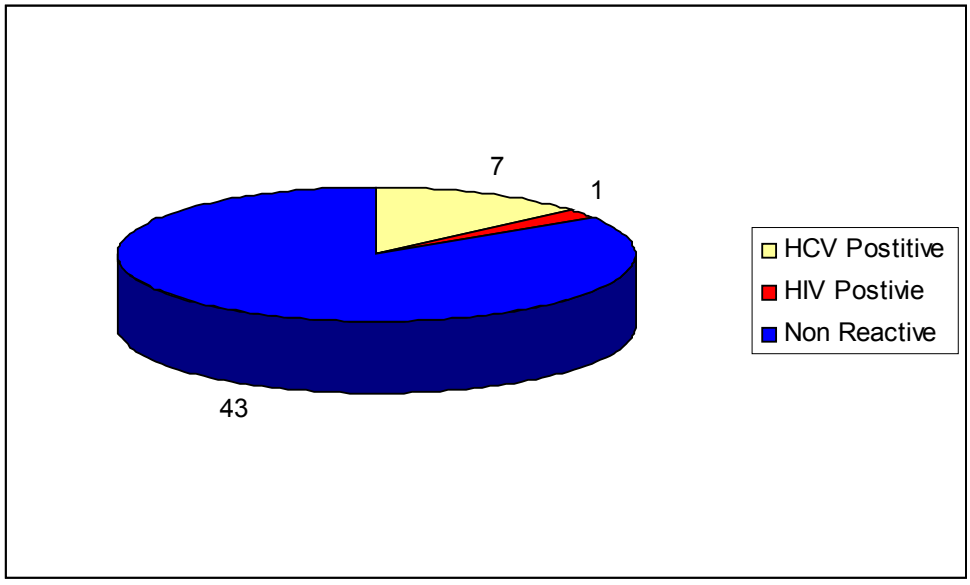
Type of reaction	Number of patients	Percent %
FNHTR	13	25.5
No Reaction	38	74.5
Total	**51**	**100.0**



Febrile non Hemolytic transfusion reaction was only acute transfusion reaction noted in the patients during the study period. Of the total 51 patients studied, 13 had febrile non hemolytic transfusion reaction on one or more occasions. No hemolytic reaction was observed during the study period.

Table 12 Transfusion Transmitted Infections

TTI Results	Number of Patients	Percent %
HCV Positive	7	13.7
HIV Positive	1	2.0
Non reactive	43	84.3



7 (13.7%) patients had HCV, one had HIV, in total of 51 patients studied. None of them had HBV, syphilis or malaria.

Table 13 Status of spleen

Splenectomy	Number of Patients	Percent %
Yes	16	31.4
No	35	68.6
Total	51	100.0

31.4% of the patients were splenectomised before the start of the study.

Alloimmunisation

A total of 51 multiply transfused thalassemia patients were studied in which none of them was found to have developed alloantibody to any of the red cell antigens.

6. DISCUSSION

Alloimmunisation to red cell antigens is one of the major complication of chronic blood transfusion

The causes of alloimmunisation in thalassemia patients are not fully understood. Various factors affect the rate of alloimmunisation, such as ethnic factors, disparity between antigenic frequency of donors and recipient, age at start of first blood transfusion, type of blood product used (leuko reduced or non-leuko reduced), number of units transfused and splenectomy.[29]

Table 14 The following table shows the prevalence of alloimmunisation among thalassemia major patients in various studies

Study	Number of patients	Percentage of Alloimmunisation
Bibi shahin shamsian et al. 2008	121	7.4
Ansari et al. 2008	80	3.75
M.N. Noor Hashina et al. 2006	58	8.6
Khalid Hassan et al. 2004	75	22.7
G. Sirchia et al. 1985	1435	5.2
Present Study	51	0

As it is seen from the table, the prevalence of red cell

alloimmunisation varies from as low as 3.75% a study by Ansari et al. to as high as 22.7% in Pakistan by Khalid et al.[30]

A low rate of alloimmunisation is attributed to homogenicity between the blood donor and the recipient population. Sirchia et al. showed a low prevalence of 5% alloimmunisation in presumed homogenous population of Greece and Italy. In the present study also 94% of the patients were homogenous population of Tamil Nadu.[31]

Rubella and model reported a lower incidence of alloimmunisation (2%) who started their transfusion before 12 months as opposed to 15% who started their transfusion after 48 months. In the present study 94% of the patients had their first transfusion before 12 months.

Blumberg et al. found that WBC reduction is associated with reduced frequency of alloimmunisation which is attributed to reduced immune activation in leukodepleted blood.[32] All the patients in the present study received leuko poor blood.

Floss et al. observed that children do not produce alloantibodies and immunological mediated transfusions are quite rare despite exposure to many red cell antigens.[33] In the present study also alloimmunisation to red cell antigen was not detected.

There was no alloantibody identified in a study by Tahhan et al on 40 Arab multitransfused children.[30] The homogeneity between the donor and recipient population, the age at which the first transfusion was given and the utility of leuko reduced products to all patients could be the reason.

The high prevalence of TTI in this study i.e. 7 patients (13.7%) for HCV and 1 patient (2%) for HIV may be due to reason that some of the patients received blood from other centers before registering into the present centre, and also possibly due to window period donations.

7. SUMMARY AND CONCLUSION

- This study was conducted on 51 registered thalassemia major patients for the presence of alloantibody to red cell antigens.
- 26 patients were males and 25 of them were females. Majority of the patients had maintained a pretransfusion hemoglobin of 7 – 9.5 gm/dl.
- 70% of the study population were below 14 yrs of age.
- 94% of the study population were ethnically identical with the donor.
- All the transfused units were leukocyte reduced.
- Most of the patients had their transfusion once every 21 days.
- None of the 51 patients developed alloantibody to any of the red cell antigens.
- Six of the patients had transfusion transmitted hepatitis C virus and one patient had HIV virus infection.

8. LIMITATIONS OF THE STUDY

1. Only 51 thalassemia major patients were enrolled at the centre where this study was undertaken. The study could be carried out on more number of thalassemia major patients.

2. Antibody screening was done by using conventional tube technique. The gel technique could be more sensitive.

9. BIBLIOGRAPHY

1. Noor Haslina MN, Ariffin N, Illuni Hayati I, Roseline H. Red cell. Red cell auto antibodies among thalassemia patients in Hospital University sains Malaysia. Singapore Med. J 2007; 48:922-925.

2. M. Sengupta thalassemia among tribal communities of India. The Inter net Journal of Biological Anthropology. 2008. Sited on 10.11.2008.

3. Khalid Hassan et al. Red cell alloimmunisation in Repeatedly Transfused Thalassemia Major patients. International Journal of pathology. 2004; 1 : 16 – 19.

4. S. Ansari, P. Voosogh and S.Moshtaghian Assessment of Frequency of Alloimmunisation and Erythrocyte Autoimmunisation in Transfusion Dependent thalassemia patients. Acta medica. Iranica, 2008; 46 : 137 – 140.

5. Hassan Myaish. Thalassemia e-med 2007. Sited on 12.12.2008.

6. W. osterag, G. Steinheider, H. Melderris Embryonic hemoglobins. 2008; 8 : 393-93.

7. Pathological basis of Disease – Robbins and Cortan – 7th edition page 634.

8. Caterina Borgna – Pignatti and Renzo Galanello. Thalassemia and related disorders: Quantitative disorders of hemoglolobin synthesis Wintrobe's clinical Hematology page 1317 – 1318.

9. K.C. Chaudhri Burden of genetic disorders in India. Indian Journal of paediatrics. 2008; 67.

10. Thalassemia International Federation (TIF). Guidelines for clinical management of Thalassemia. April 2000.

11. C.B. Modell Hemoglobinopathies. The patho physiology of beta – thalassemia major. J. Clin. Path 2000; 8.12.18.

12. Modell C.B, Berdoukas VA. The clinical approach to thalassemia Grune and sratton, New york. 1994.

13. F.A. Rice, Thalassemia. ART CLS 1996.

14. Fucharoen S, Wini cha goon P, Thonglairuan V, Siriboon W, Sae-Ngow B. Laboratory diagnosis for thalassemia. Ann Med. Singapore. 1989; 18: 424 – 430.

15. The Thalassemia syndromes. V.J.wealtherall and J.B. Clegg. 4th edition page 633 – 635.

16. Schone wille H, Haak HC, Vanzift AM. Alloimmunisation after blood transfusion in patients with hematologic and oncologic disease. Transfusion 1999; 39 : 763 – 771.

17. Anne F.Eder, Linda A. Chamber. Non infection complications of Blood Transfusion. Arch pathol Lab. 2007; 131. 708 – 718.

18. Ness Pm, Shirey RS, Thoman Sk, Bucksa. The differentiation of delayed hemolytic and delayed serologic transfusion reactions; incidence, long term serological findings and clinical significance. Transfusion 1990; 30. 688 – 693

19. National Heart Lung and Blood Institue, National Institute of Health. The Management of sickle cell Disease.4th edition. Bethesda Md: National Heart Lung, and Blood Institute; 2002 NIH Publication No : 02 – 2117.

20. George B. Schreiber et al. The Risk of Transfusion – Transmitted viral Infections. The New England Journal of medicine. 1996; 334 : 1685 – 1690

21. Murugan Ragavan and Paul E. Marik, Anemia Allogenic Blood transfusion, and Immunomodulation in the critically III.

American college of chest physicians. 2005; 127:295 – 307

22. Green NS Yersinia infections in patients with homozygous beta-thalassemia associated with iron over load and its treatment. Pediatr Hematol oncol. 1992; 9: 247 – 254

23. Renzo Galanello. Deferiprone in the treatment of transfusion – dependent thalassemia: a review and Prespective. Therapeutics and clinical risk management 2007; 3

24. Anita saxena Growth Retardation in thalassemia major patients. Int J Hum Gene. 2003; 4 : 237 – 246

25. Hawsawi ZM, Humnaida T1, Ismail GA, Splenectomy in thalassemia major: Experience at madina materity and children Hospital, saudi Arabia. Ann Trop paediatr. 2001; 21: 155 – 158.

26. M de Montalebert et al. partial sptenectomy in homozygous β thalassemia. Archeives of Disease in childhood. 1990; 65: 304 – 307.

27. R. Keith Hurphries et al. 5-Azacytidine Acts Directly on Both Erythroid precursors and progenitors to Increase production of Fetal Hemoglobin. J, clin Invest. 1985; 75: 547- 557.

28. Punam Malik and paritha I. Arumugam Gene therapy for β -

thalassemia. Hematology, 2005; 45 – 50.

29. Bibi Sahin et al. Frequency of Red ced cell Alloimmunisation in patients with β - Thalassemia in an Iranian Referral Hospital. Iran J pediatr 2008; 18. 149 – 153

30. Tahhan HR Holbrook CT Braddy LR, Brewel LD, Christie JD Antigen matched donor blood in Transfusion management of patients with sickle cell disease. Transfusion 1994;34 562 – 569.

31. G. Sirchia et al. Red cell alloantibodies in thalassemia major. Results of an Italian co-operative study. Transfusion 1985; 25:110 – 112

32. Blumberg et al. should chronic transfusion be matched for antigens other than ABO an Rh (D)? vox sang 1984; 47: 205-208.

33. Floss et al. multiple transfusion fail to provoke antibodies against blood cell antigens in human infants. Transfusion 1986; 26: 419-422.

10. APPENDIX

10.1 THESIS PROFORMA

Name	:
Address	:
Phone no	:
Age	:
Sex	:
Age at diagnosis	:
Age at starting of Transfusion	:
Place of diagnosis	:
Blood Group	:
Pre Transfusion Hemoglobin	:
Iron Chelation therapy: Yes /no	:
Splenectomy: Yes / no	:
H/O Transfusion reactions	:
RBC alloantibody screen	:
Antibody Identification	:

CONSENT

I confirm that I read and understood the information dated for

Above research study dated ----------and I received chance to ask the questions.

My participation in this study is voluntary and I know that I am free to withdraw from the study at any time, without giving any reason and without affecting of my medical care or legal rights.

I agree not to restrict or interfere any data or results those are used properly those are obtained from this study.

I agree to participate in this research study for the above listed purpose.

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patient's Name (print)

Age of the patient :----------

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Signature of Parent /guardian Date

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Signature of the person who obtains consent Date

Subject ID No.

Subject Initials

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S. No	Name	Age / Sex	Blood Grp	Ethnicaty	Age at Diag., start of 1st trans.	Transfusion Regimen Frequency	Present Clinical Status	Transfusion Reactions	Increased Blood Req.	Splenectemy	TTI Results	Hb%	Alloantibody Scrren
1	Punniasloksamal	6 yrs / M	0 +	tamil nad	3 months	regularly once 21 days	Normal	No	No	No	Neg	Hb-8.5 g /dl	Nil
2	Hemadurga	7 yrs / f	B +	tamil nad	3 months	Regular once 21 days	Normal	No	No	No	Neg	Hb-7 g /dl	Nil
3	Jeyavedan	6 yrs /M	B +	tamil nad	3 months	Regular 21 days		No	No	No	Neg	Hb-7.5 g /dl	Nil
4	Sugan	3 yrs / M	B neg	tamil nad	3 months	Regular 21 days	Normal	No	No	No	Neg	Hb-8 g /dl	Nil
5	Shiva	7 yrs / M	A neg	tamil nad	6 months	Regular 21 days	Normal	Frequent FNHTR	No	No	**HCV +**	Hb-8.5 g /dl	Nil
6	Sandhiya	5 Yrs / F	B Pos	tamil nad	3 Yrs	Regular 21 days	Normal	No	No	No	neg	Hb- 7g /dl	Nil
7	Madhu	14 Yrs / M	AB Pos	tamil nad	6 months	Regular one month	Growth retard	no	No	At 5 yrs of age	neg	Hb- 7.6g /dl	Nil
8	Sahana	12 Yrs / F	B Neg	tamil nad	6 months	Regular 21 days	Growth retard	no	No	No	neg	Hb- 7g /dl	Nil
9	Anaga	13 yrs / F	B pos	tamil nad	6 months	Regular 21 days	Normal	no	No	No	neg	Hb- 7.8 g /dl	Nil
10	Fazila Banu	27 yrs / F	B Pos	tamil nad	one year	Regular 21 days		No	No	No	**HIV +**	Hb- 8.5 g /dl	Nil
11	Suganya	10 yrs / F	O Pos	tamil nad	6 months	Not under regular	Normal	No	NO	At 5 yrs of age	Neg	Hb- 8.5 g /dl	Nil
12	Manigandan	6 yrs / M	O Pos	tamil nad	9 months	Regular 21 days	splenomegaly	no	No	No	Neg	Hb- 6.5 g /dl	Nil
13	Asharaf	8 yrs / M	A pos	tamil nad	1 1/2 yrs	Regular 21 days	Anemia	no	No	No	Neg	Hb- 6 g /dl	Nil
14	Yogeswaran	8 yrs / M	O Pos	tamil nad	3 months	Regular 21 days	normal	No	No	No	Neg	Hb- 7.6g /dl	Nil
15	Aneez Nazreen	6 Yrs / F	A1B Pos	tamil nad	2 yrs	Regular 21 days	Normal	No	No	No	Neg	Hb- 8.3 g /dl	Nil
16	Sanjay	20 yrs / M	O Pos	Rajasthan	4 months	Regular 21 days	Growth retard Cardi abnor	No	No	At 6 Yrs of Age	Neg	Hb- 7.8 g /dl	Nil
17	Aravind Srinivas	18 yrs / M	B post	tamil nad	3 months	Regular 21 days	Growth retard cardiac abnor	FNHTR +	No	in 1993	**HCV +**	Hb- 7.5 g /dl	Nil
18	Salman	19 yrs / M	O Pos	tamil nad	6 months	Regular 21 days	Growth retard	FNHTR +	No	At 6 yrs of Age	Neg	Hb- 7.5 g /dl	Nil
19	Katheeja	6 Yrs / F	B post	tamil nad	5 Months	Regular 21 days	Frontal bossing	FNHTR +	No	No	Neg	Hb- 5 g /dl	Nil
20	Asithir	one yr / F	B Post	tamil nad	3 months	Regular 21 days	Normal	No	No	No	Neg	Hb- 7.8 g /dl	Nil
21	Lawanya Sri	1 1/2 yr / F	O Pos	tamil nad	5 months	Regular one month		No	No	No	Neg	Hb- 8.5 g /dl	Nil
22	Sathish	19 yrs / M	B Pos	tamil nad	6 months	Regular 21 days	Growth retard cardiac abnor	No	No	yes / 11.5.2006	**HCV +**	Hb-7 g /dl	Nil
23	Mahalakshmi	6 Yrs / F	B Pos	tamil nad	6 months	Regular 21 days	Normal	No	No	No	Neg	Hb-7 g /dl	Nil
24	Rajkumar	3 yrs / M	A Pos	tamil nad	6 months	Regular 21 days	Normal	No	No	No	Neg	Hb-8.5 g /dl	Nil
25	Eyal Raj	17 / M	B Pos	tamil nad	3 years	Not regular	Growth retard hypoparathyroid	No	No	At 10 yrs of age	Neg	Hb-7.5 g /dl	Nil

S. No	Name	Age / Sex	Blood Grp	Ethnicaty	Age at Diag., start of 1st trans.	Transfusion Regimen Frequency	Present Clinical Status	Transfusion Reactions	Increased Blood Req.	Splenectemy	TTI Results	Hb%	Alloantibody Scrren
26	Nikleswaran	9 yrs / M	O pos	tamil nad	6 months	Regular 21 days	Normal	No	No	No	neg	Hb-8 g /dl	Nil
27	Krishna	2 yrs / M	O pos	tamil nad	6 months	Regular 21 days	Normal	No	No	No	neg	Hb- 7.5 g /dl	Nil
28	Ayyappan	14 yrs / M	A pos	tamil nad	3 months	Regular one month	Normal	FNHTR +	No	At 4 yrs of age	**HCV +**	Hb- 8.3 g /dl	Nil
29	Vijayalakshmi	25 yrs / F	A1 pos	tamil nad	3 months	Regular 3-4 months	Growth retard Diabetis	FNHTR +	No	At 7 yrs of age		Hb- 6.5 g /dl	Nil
30	Paneerselvam	28 yrs / M	B pos	tamil nad	7 month	Regular 21 days	Hepatomegaly Growth retard cardiac abnor	FNHTR +	No	At 5 yrs of age	HCV+	Hb-7.5g/dl	Nil
31	Thenmozhi	17 yrs / F	O pos	tamil nad	3 months	Regular 6 months	Growth retard Hypothyroid		yes	yes at 2003	**HCV +**	Hb- 8 g /dl	Nil
32	Rahul	9 yrs / M	B pos	tamil nad	6 months	Regular 21 days	SPLEEN +	FNHTR	yes	no	neg	Hb- 6 g /dl	Nil
33	Shankari	3 yrs / F	B pos	tamil nad	3 months	Regular one month	Normal	No	No	No	neg	Hb- 8.4 g /dl	Nil
34	Banushree	3 yrs/ F	O pos	tamil nad	9 months	Regular one month	Normal	No	No	No	neg	Hb- 8 g /dl	Nil
35	Thamirabarani	8 yrs / F	O pos	tamil nad	one year	Regular one month	Normal	FNHTR	yest	at 2007	neg	Hb- 7. g /dl	Nil
36	Leema Elizabeth	8 yrs / F	A1 pos	tamil nad	4 yrs	Regular one month	Normal	No	No	No	neg	Hb- 7.8 g /dl	Nil
37	Vaishnavi	11 yrs / F	O pos	tamil nad	2.5	Regular 21 days	spleenomegaly	No	No	No	neg	Hb- 8.2 g /dl	Nil
38	Navaneetha krishnan	18 yrs /M	O pos	tamil nad	7 months	Regular 21 days	Growth retard Diabetis	No	No	at 3 1/2 yrs	neg	Hb- 6.8 g /dl	Nil
39	Sowndarya	7 yrs / F	O pos	tamil nad	4 yrs	Regular one month	Normal	No	No	yes / 6.11.2007	neg	Hb- 7.6 g /dl	Nil
40	Daniel Raj	1 1/2 / M	O pos	tamil nad	7 months	Regular one month	Normal	No	No	No	neg	Hb- 7.4 g /dl	Nil
41	Yogeswari	8 yrs / M	O pos	Maharastra	3 months	Regular 21 days	Normal	No	Yes	No	Neg	Hb- 8 g /dl	Nil
42	Vignesh	7 yrs / M	B pos	tamil nad	6 months	Regular 21 days	Normal	No	No	No	neg	Hb- 8.4 g /dl	Nil
43	Durga	4 yrs / F	O pos	tamil nad	5 months	Regular 21 days	Normal	No	No	No	Neg	Hb- 7.6 g /dl	Nil
44	Kavya Shree	2 yrs / F	A pos	tamil nad	6 months	Every 35 days	Normal	No	No	No	Neg	Hb- 7.6 g /dl	Nil
45	Sundar Sham	8 yrs / M	O pos	tamil nad	4 months	Regular 30 days	Normal	No	No	No	neg	Hb- 7.2 g /dl	Nil
46	Priya	12 yrs / F	B pos	tamil nad	5 months	Regular 21 days	Normal	No	No	No	neg	Hb- 8.2 g /dl	Nil
47	Mahesh	8 yrs / M	O pos	tamil nad	3 months	Regular 21 days	Normal	No	FNHTR+	No	neg	Hb- 7.6 g /dl	Nil
48	Jeeva	9 yrs / M	B pos	tamil nad	tamil nad	Regular one month		No	No	No	neg	Hb- 8.2 g /dl	Nil
49	Suba	6 yrs / F	AB Pos	tamil nad	tamil nad	Regular 21 days	spleenomegaly	No	No	No	neg	Hb- 6.4 g /dl	Nil
50	Kumar	14 yrs / M	A Pos	tamil nad	3 months	Regular one month	Growthh retard	No	No	yes at 4 yrs	neg	Hb- 8 g /dl	Nil
51	Alamelu	12 yrs / F	O pos	tamil nad	4 months	Regular 21 days	Normal	No	No	yes at 5 yrs	neg	Hb- 7.2 g /dl	Nil

10. **i-iii**